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14. ABSTRACT: In this study, we generated a novel mouse model of human breast cancer based on a recurrent chromosomal translocation that produces the TEL-NTRK3 fusion oncogene, as the initiating mutation in human secretory breast carcinoma. In this model, we created a Cre-lox conditional knockin allele (TN) to express the TN fusion protein from the endogenous Tel locus in a tissue-specific manner. When TN is activated in mammary glands by whey acidic protein (WAP) promoter-driven Cre, all female mice exhibit extensive lobuloalveolar hyperplasia and develop multifocal mammary tumors as early as 4-month of age. Using the Cre-lox genetic marking approach coupled with a conditional Rosa26-lacZ reporter, we identified putative target cells of TN, a population of transient mammary epithelial cells (MECs) that appear only during estrus in wild type virgin females, but become premalignant hyperplastic MECs once TN is activated by WAP-Cre. Additional genetic/epigenetic changes may eventually convert them to cancer. Thus, our model is useful for studying target cells of breast cancer and provides a preclinical system for testing novel therapies for breast cancer.				
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INTRODUCTION:

Breast cancer will affect one in nine women (1). ~10% of breast cancer cases are hereditary, often caused by mutation of tumor suppressor genes, such as *BRCA1* (2). However, the majority of breast cancer cases are sporadic, and mutations in *BRCA1* are rarely identified in non-hereditary breast cancer (3). In fact, mechanisms of oncogenesis in sporadic breast cancer remain largely unknown, in part due to the cytogenetic complexity often associated with sporadic breast cancer. This has largely precluded the discovery of primary genetic events that lead to transformation of breast epithelium (4).

Several years ago, a recurrent translocation involving human chromosomes 12 and 15 was identified in patients with secretory breast carcinoma (SBC) (4). This translocation brings together coding regions of the *TEL* (also known as *ETV6*, located on chromosome 12) and *NTRK3* (also called *TRKC*, located on chromosome 15) genes such that *TEL* contributes an oligomerization domain (the PNT domain) to the protein tyrosine kinase (PTK) domain of *NTRK3*. Remarkably, this fusion gene has been found in virtually all cases of human SBC (4). The *TEL-NTRK3* fusion protein is a potent oncoprotein in transforming fibroblasts and cultured mammary epithelial cells (MECs) (4). The identical fusion protein is also found in two pediatric cancers, including congenital mesoblastic nephroma and infantile fibrosarcoma (5-7), and in one case of acute myelogenous leukemia (8).

The occurrence of such a chromosome translocation as the sole cytogenetic alteration in SBC provides an excellent opportunity to study pathways underlying the initiation and progression of breast cancer, and how these pathways may be targeted therapeutically. The purpose of this research is to generate mice that faithfully express the *TEL-NTRK3* fusion gene specifically in their mammary epithelial cells as a means of creating a novel mouse model of human breast cancer. Such a model (based on the initiating genetic event in one type of human breast cancer) will enhance our understanding of cellular pathways affected in breast cancer and provide a new system for preclinical testing of potential new therapies.

Specifically, we have proposed three aims for this research, including (1) By gene targeting in mouse ES cells create a conditionally-expressed allele of the *TEL-NTRK3* fusion gene within the endogenous *Tel* locus (designated lox-STOP-TN, will refer to as *TN* from here on); (2) Generation of germline transmitting chimeras and establishment of a colony of *TN* mice; (3) Breeding of *TN* mice to mouse strains that express Cre-recombinase in mammary epithelium to active expression of the *TN* oncogene. We reasoned that if aims 1-3 were to be accomplished, it would be highly likely that mammary tumors would be observed, and we would then compare the pathology and behavior of mouse mammary tumors with that of sporadic human breast cancer.

In this final report of the award, we will describe the successful accomplishment of all three aims we proposed previously. We will show that this novel mouse model provides unique insights into the pathogenesis of breast cancer and also provides a new preclinical system for testing novel therapies for breast cancer.

BODY:

Specific aim 1: By gene targeting in mouse ES cells create a conditionally-expressed allele of the *TEL-NTRK3* fusion gene within the endogenous *Tel* locus – Status: accomplished.

We generated a Cre-Lox conditional *Tel-NTRK3* (*TN*) knock-in allele that expresses a mouse Tel-human NTRK3 fusion protein (*TN*) from the endogenous *Tel* locus in a tissue-specific manner (Fig. 1A). Specifically, we knocked in the portion of human *NTRK3* cDNA encoding the PTK domain (as found in SBC patients) into mouse *Tel* locus (Fig. 1A). In the t(12;15)(p13;q25) translocation, the breakpoint in the human *TEL* locus is located in the intron between exon 5 and 6. Therefore, we introduced *NTRK3* cDNA into the equivalent exon 6 of mouse *Tel* locus (Fig. 1A). The resulting allele is designed to fuse the first five exons of mouse *Tel* in-frame with the knocked-in human *NTRK3* cDNA. Since the same t(12;15)(p13;q25) translocation is found in multiple tumor types and the resulting *TN* fusion protein appears to be a potent oncoprotein, we reasoned that constitutive expression of this fusion gene might lead to embryonic lethality (which turned out to be the case). Hence, we designed this knockin allele as a “conditional” allele by inserting a transcriptional terminator sequence (“Stopper”, Fig. 1A) into the intron immediately upstream of the knocked-in *NTRK3* cDNA. The “stopper” sequence was flanked by *loxP* sites, together with the *Neomycin* (*Neo*) selection marker. The resulting *Tel-NTRK3* (*TN*) conditional knockin allele will not be expressed until the “stopper” cassette is excised by Cre recombinase. When coupled with Cre recombinase-expressing transgenic lines under the control of mammary-specific promoters, *TN* oncoprotein will be expressed from the endogenous *Tel* promoter specifically in mammary epithelial cells.

The *TN* conditional knockin targeting vector was electroporated into mouse CJ7 ES cells. Correctly targeted ES cell clones were screened by Southern blot (Fig. 1B). To examine if excision of the “stopper” cassette from the *TN* conditional allele can lead to the expression of the *TN* fusion protein, we tested it in knockin ES cells carrying this conditional allele. The correctly targeted knockin ES cells were transiently transfected with a plasmid expressing a GFP-Cre fusion protein(9). GFP⁺ ES cells were FACS (fluorescence activated cell sorting)-sorted and plated out. Single colonies were picked up and expanded, and tested by PCR for the removal of the “stopper” cassette together with the *Neo* selection marker. The parental *TN* knockin ES cells and the derived “stopper”-removed ES cells were then tested by both RT-PCR and Western blot to check the expression of the *TN* fusion at both the transcript and protein level (Fig. 1C-D). As shown in Figure 1C, the parental ES cell line (TN28) has slightly leaky expression of the *TN* allele at the transcript level, but removal of the “stopper” cassette greatly increased the expression of this conditional allele. At the protein level, no detectable *TN* fusion protein signal was seen in the parental knockin ES cell line. The expression of the *TN* fusion protein was clearly visible from the two derived ES lines without the “stopper” cassette (Fig. 1D). These results suggest our *TN* conditional knockin allele works as we initially designed.

Specific aim 2: Generation of germline transmitting chimeras and establishment of a colony of *TN* mice – Status: accomplished.

Two ES clones carrying the correctly targeted conditional knockin *TN* allele were injected into mouse blastocysts by standard procedures. Both clones gave rise to male chimeras with high

chimerisms. These chimeras were then bred to C57 wild type (WT) females and the *TN* conditional allele was germline-transmitted. A colony of *TN* mice was established from the germline-transmitted *TN* allele. *TN* mice, without Cre-mediated activation of the *TN* allele, are largely indistinguishable from their WT littermates, and can be easily maintained as heterozygotes (the *TN* allele was knocked-in to one of the two *Tel* loci and disrupted the corresponding *Tel* allele, thus can not be maintained as *TN/TN* homozygotes, because *Tel*^{-/-} is embryonic lethal).

Specific aim 3: Breeding of *TN* mice to mouse strains that express Cre-recombinase in mammary epithelium to active expression of the *TN* oncogene – Status: accomplished.

To activate TN expression specifically in mammary epithelium, we initially planned to use two mammary-gland-specific Cre mouse lines, *MMTV-Cre* and *WAP-Cre*(10). Both lines have been used successfully to develop mouse models of human breast cancer(11-13). Unfortunately, when we used *MMTV-Cre* to activate TN expression, all *MMTV-Cre;TN* mice developed a lethal myeloproliferative disease (MPD) within several weeks after birth, apparently due to leaky expression of this Cre line in blood cells. This is also the case for an interferon-inducible Cre line, *Mx-Cre*(14) (Fig. 2). In *Mx-Cre;TN* mice, even before induction of Cre expression by poly(I)-poly(C) injection, the animals have already developed MPD due to leaky expression of Cre in hematopoietic cells. The lethal MPD developed in mice due to expression of TN in hematopoietic system is consistent with the similar disease developed in a murine bone marrow transplantation model for TN reported previously(15).

When TN expression is specifically activated in mammary tissues by *WAP-Cre* (Fig. 3M), ALL *WAP-Cre;TN* females developed multi-focal mammary tumors. The expression of whey acidic protein (WAP) is greatly elevated only during pregnancy and lactation(16). Thus, we initially reasoned that *WAP-Cre;TN* female mice needed to go through rounds of pregnancies in order to activate Cre expression in them. In addition, it is well-known that pregnancy has a major impact on breast tumorigenesis(17). In most of the current mouse models of breast cancer, pregnancy typically accelerates tumorigenesis. As expected, *WAP-Cre;TN* female mice developed mammary tumors after 1-2 rounds of pregnancies. However, later we discovered that even virgin *WAP-Cre;TN* developed tumors. In fact, all virgin *WAP-Cre;TN* female mice developed multi-focal mammary tumors as early as 4-month of age, this was preceded by extensive mammary lobuloalveolar hyperplasia (Fig. 3A,E). The mammary tumors in *WAP-Cre;TN* animals were heterogeneous, with different morphologies and rates of tumor progression (Fig. 3C,D,G-L). Many were positive for estrogen receptor, the mammary progenitor marker keratin 6 (K6, (18)), and the basal cell marker keratin 5 (k5) (Fig. 4). The majority of them were invasive and transplantable when subcutaneously injected into immunodeficient male or female mice. The rates of tumor re-growth following transplantation correlated with the rates of progression of their corresponding primary tumors. Due to the relatively short latency of these tumors, most *WAP-Cre;TN* mice failed to show any signs of metastasis. In rare occasions, tumor cells metastasized to lymph node (Fig. 3J) and lung (Fig. 3L). FACS analysis showed that most of the *WAP-Cre;TN*-derived mammary tumor cells were positive for both CD24 and Sca-1. In fact, mammary glands from mature virgin *WAP-Cre;TN* females that have not developed visible tumors have already accumulated more CD24⁺Sca-1⁺ cells than WTs (Fig. 5), suggesting that these might represent a population of abnormal cells as a result of TN expression. Similarly, in

mammary tumors derived from *MMTV-Wnt-1* mice, Sca-1 is also present in a large fraction of tumor cells(18). In contrast, tumors induced by transgenic expression of Neu or PyMT do not express Sca-1(18). In *MMTV-Wnt1* (but not in *MMTV-Neu*) hyperplastic mammary glands, the Lin⁻CD29^{hi}CD24⁺ mammary stem cell (MaSC) subpopulation is significantly expanded, suggesting that MaSCs might be direct targets of *Wnt-1*(19). We analyzed the percentage of Lin⁻CD29^{hi}CD24⁺ (and the similar Lin⁻CD49f^{hi}CD24⁺, (20)) MaSC subpopulation in *WAP-Cre;TN* hyperplastic mammary glands and found is not changed compared to WT mammary glands (Fig. 5). These data suggest that mammary tumors in *WAP-Cre;TN* virgins may develop from a distinct population of mammary progenitors, but not directly from MaSCs.

Although the expression of WAP is greatly increased only during pregnancy and lactation, in mature virgin female mice, previous studies have shown that the *WAP* locus is also activated in a small population of alveolar and ductal MECs during estrus. But this expression is transient(16, 21, 22). Further studies using the conditional *Rosa-lox-lacZ* reporter bred to *WAP-Cre* mice showed that these *WAP-Cre*⁺ MECs (thus also *lacZ*⁺) do not persist in normal mice during diestrus. Since in this Cre-mediated genetic marking experiment, *lacZ* expression in the *WAP-Cre*⁺ cells, once activated, is no longer dependent upon the expression of Cre. They should still be *lacZ*⁺ if they persist in the tissue, even when mice are not at estrus. In addition, all progeny cells derived from them should also be *lacZ*⁺. But this is not the case in *WAP-Cre;Rosa-lox-lacZ* virgin females, suggesting the *WAP-Cre*⁺ MECs do not proliferate extensively and possibly die through apoptosis(23-25).

Since *WAP-Cre;TN* virgin female mice develop multi-focal mammary tumors with 100% penetrance, and the above-mentioned transient *WAP-Cre*⁺ MECs are probably the only cells in these mice that express Cre and activate TN, we reasoned that these cells may be direct targets of TN transformation. To test this hypothesis, we bred a similar *Rosa-lox-lacZ* reporter(26) to *WAP-Cre;TN* mice and generated *WAP-Cre;TN;Rosa-lox-lacZ* triple transgenic females. If the hypothesis is correct, we should see *lacZ*⁺ hyperplastic MECs in mammary glands, followed by *lacZ*⁺ mammary tumors. This is precisely what is observed (Fig. 6).

Our preliminary studies on the *WAP-Cre;TN* breast cancer model support the idea that the transient MECs appeared during mouse estrus in virgin females, most likely representing a transient wave of mammary progenitors, are potential targets of TN. These cells normally disappear by apoptosis in a process similar to involution. Activation of TN in these cells likely rescues them from apoptosis and meanwhile provides proliferative signals to them. Rare TN-activated cells acquire additional genetic/epigenetic changes, which lead to carcinoma *in situ*, and eventually invasive mammary tumors (Fig. 7).

KEY RESEARCH ACCOMPLISHMENTS:

- Generated the *Tel-NTRK3 (TN)* conditional knockin ES cell line.
- Established a colony of germline-transmitted *TN* conditional knockin mice.

- Derived a novel mouse model of human breast cancer by activating TN in mammary glands by Cre recombinase under the control of the Whey acidic protein (WAP) promoter.

REPORTABLE OUTCOMES:

1. Dana-Farber/Harvard Cancer Center “Stem Cells & Tumor Microenvironment” symposium 2005: invited speaker (Stuart Orkin) – “Identifying the target cell in one form of breast cancer using gene targeted mice”.
2. Third annual symposium of the Dana-Farber/Harvard Cancer Center program in breast cancer 2006: poster – “Identifying the target cell in one form of breast cancer using gene targeted mice”.
3. Dartmouth Medical School Breast Cancer Program annual retreat 2006: invited speaker (Zhe Li) – “Identifying the target cell in one form of breast cancer using gene targeted mice”.

CONCLUSIONS:

In summary, we have created mice in which an initiating event in human breast cancer, the expression of the Tel-NTRK3 fusion protein, is recapitulated. The conditional *Tel-NTRK3* mice (when interbred with *WAP-Cre* transgenic mice) develop multifocal breast cancer with antecedent mammary epithelial cell hyperplasia. This new model of breast cancer should permit assessment of the nature of the breast cancer target cell and its relationship to mammary epithelial stem and progenitor cells and tumor stem cells; the role of insulin-like growth factor receptor signaling in oncogenesis *in vivo*; gene expression signatures in common with human breast cancer; and the use of pharmacological inhibition of TRK in preclinical studies.

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APPENDICES:

None.

SUPPORTING DATA:

Figure 1.

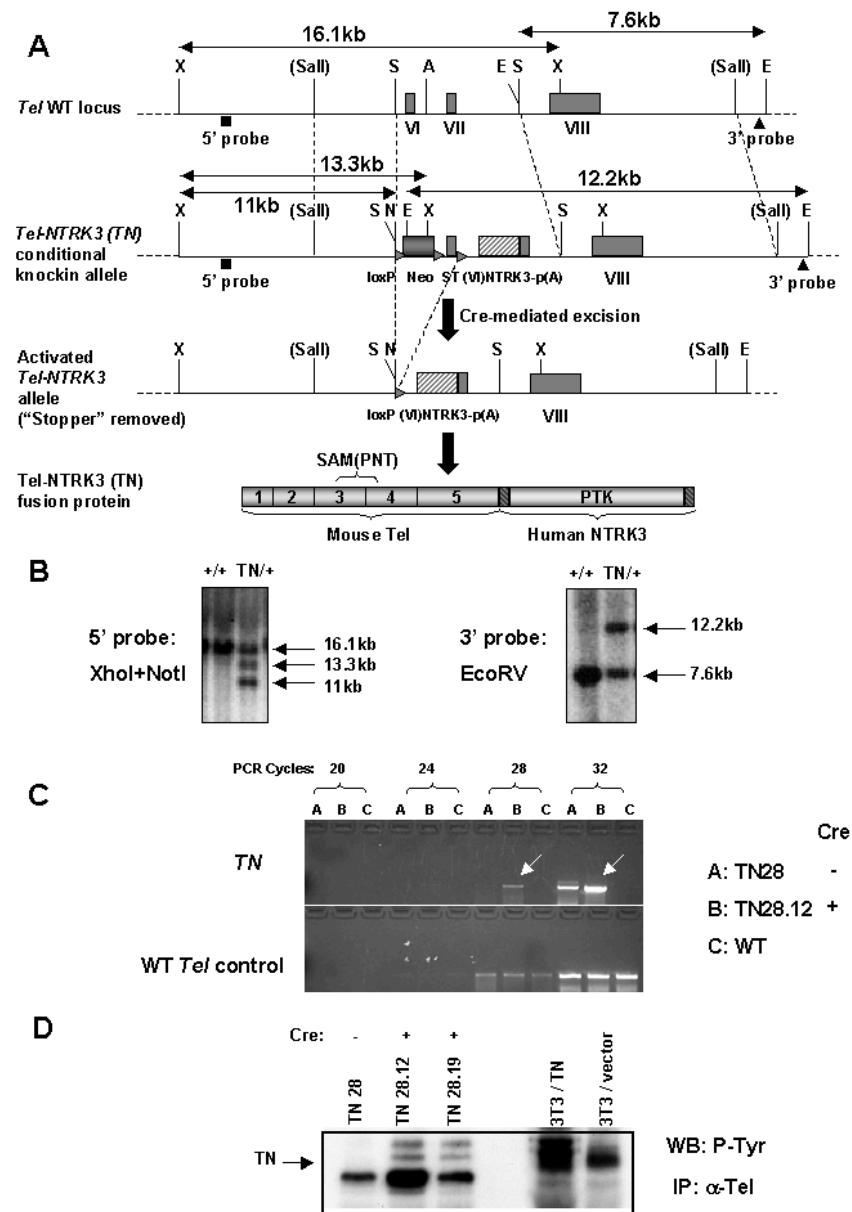


Figure 1. Generation of the *Tel-NTRK3* (TN) conditional knockin allele. A: Schematic diagram of the TN conditional knockin allele. The Southern blot screening strategies for correctly targeted ES cell clones and the 5' and 3' probes are shown. Cre-mediated excision of the floxed Stopper (ST) and Neo cassettes leads to the activation of the TN fusion gene. X: *Xba*I; S: *Spe*I; A: *Apal*; E: *Eco*RV. B: Southern blot to screen for targeted ES clones with correct 5' and 3' junctions. The 5' probe recognizes a 16.1kb *Xba*I fragment from the WT *Tel* allele, a 13.3kb *Xba*I fragment and a 11kb *Xba*I+*Not*I fragment from the TN allele. The 3' probe recognizes a 7.6kb *Eco*RV fragment from the WT *Tel* allele and a 12.2kb *Eco*RV fragment from the TN allele. C-D: TN is expressed in targeted ES cells after Cre-mediated excision of the "stopper". C: RT-PCR, the expression level of the other endogenous *Tel* allele was used as the loading control. D: Western blot (performed by Poul Sorensen's lab). The cell lysates were immunoprecipitated by anti-Tel antibody and then detected by antibody against phospho-tirosine. 3T3 cells retrovirally transduced by a TN expressing vector were used as the positive control.

Mx-Cre;TN

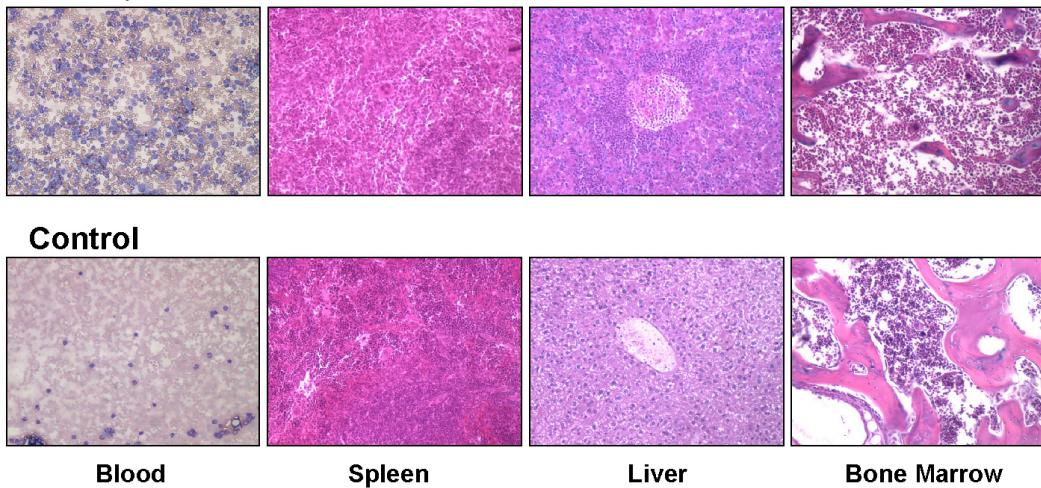


Figure 2: Postnatal activation of *TN* in hematopoietic cells (usually due to leaky expression of Cre in these cells) leads to a lethal myeloproliferative disease. Shown here is an example from *Mx-Cre;TN* mice.

Figure 3.

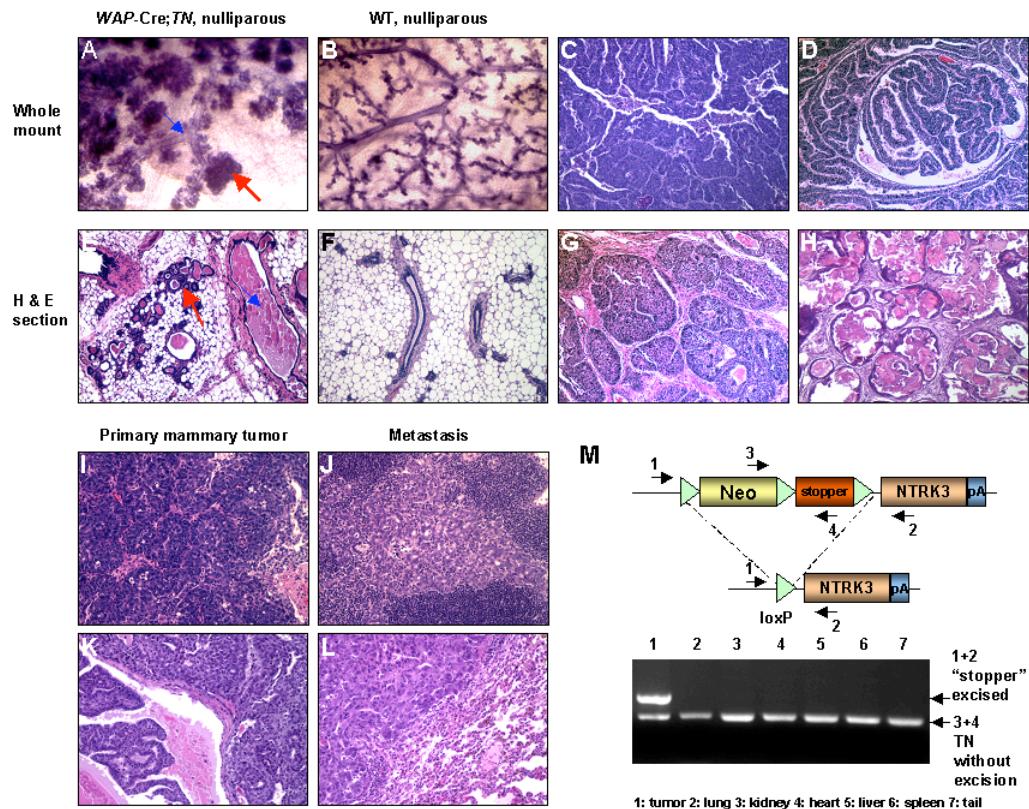


Figure 3: Activation of TN specifically in mammary glands by *WAP-Cre* leads to alveolar hyperplasia and mammary tumors. A-B,E-F: Mammary glands of virgin *WAP-Cre;TN* females (A,E) exhibit extensive lobuloalveolar hyperplasia. Red arrows: alveolar hyperplasia. Blue arrows: dilated ducts and accumulation of secretions within ducts. C-D,G-L: Hematoxylin & eosin (H&E) sections of mammary tumors developed in *WAP-Cre;TN* females. I and J were from the same mouse. I shows a mammary tumor and J shows metastasis in a lymph node. Similarly, K and L were from the same mouse. K shows a mammary tumor and L shows metastasis in lung. M: PCR strategy to show excision of The Neo-Stopper cassette only in mammary tumors in *WAP-Cre;TN* animals.

Figure 4.

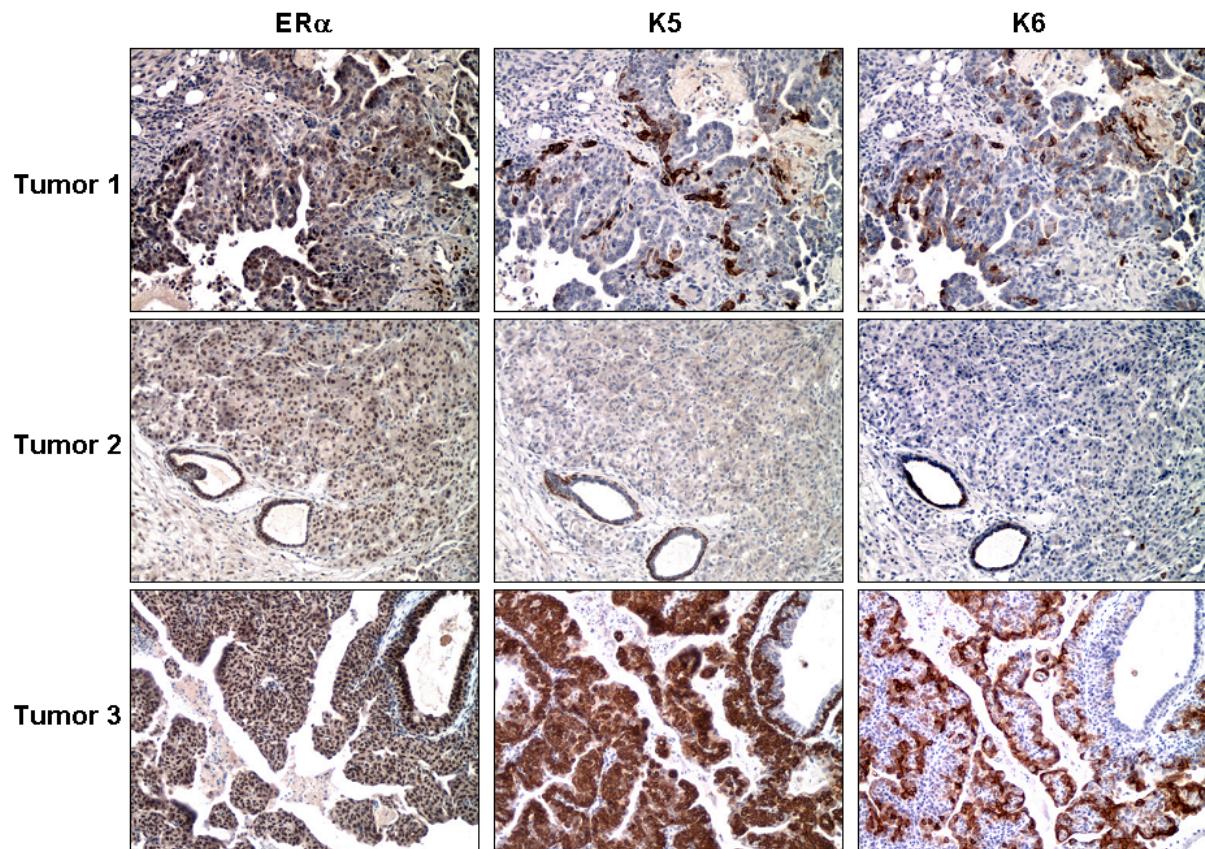


Figure 4. Immunohistochemistry to detect estrogen receptor α (ER α), keratin 5 (K5), and keratin 6 (K6) in 3 tumors from *WAP-Cre;TN* females. All three tumors are positive for ER α . Tumor 1 contains some K5 $^+$ and K6 $^+$ cells. Tumor 2 has almost no K5 $^+$ and K6 $^+$ cells. Majority of cells in tumor 3 are positive for K5, some are positive for K6.

Figure 5.

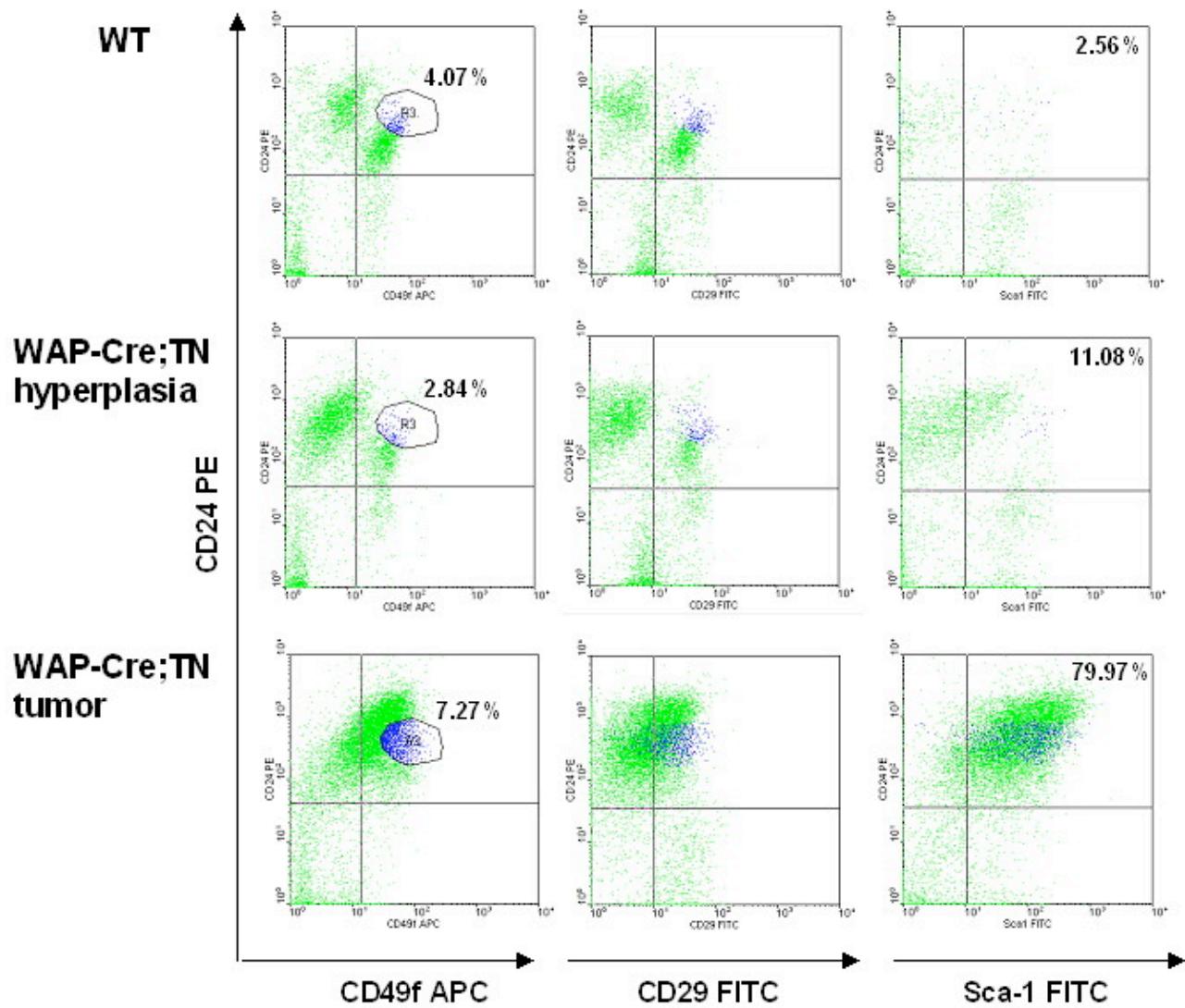


Figure 5: FACS analysis of *WAP-Cre;TN* hyperplasia and tumor. Gate R3 (blue) represents Lin⁻ CD49f^{hi}CD24⁺ mammary stem cell (MaSC) subpopulation. Note in *WAP-Cre;TN* hyperplastic mammary glands and mammary tumors, the MaSC subpopulation (blue) does NOT show significant changes. However, the CD24⁺Sca-1⁺ subpopulation is significantly expanded.

Figure 6.

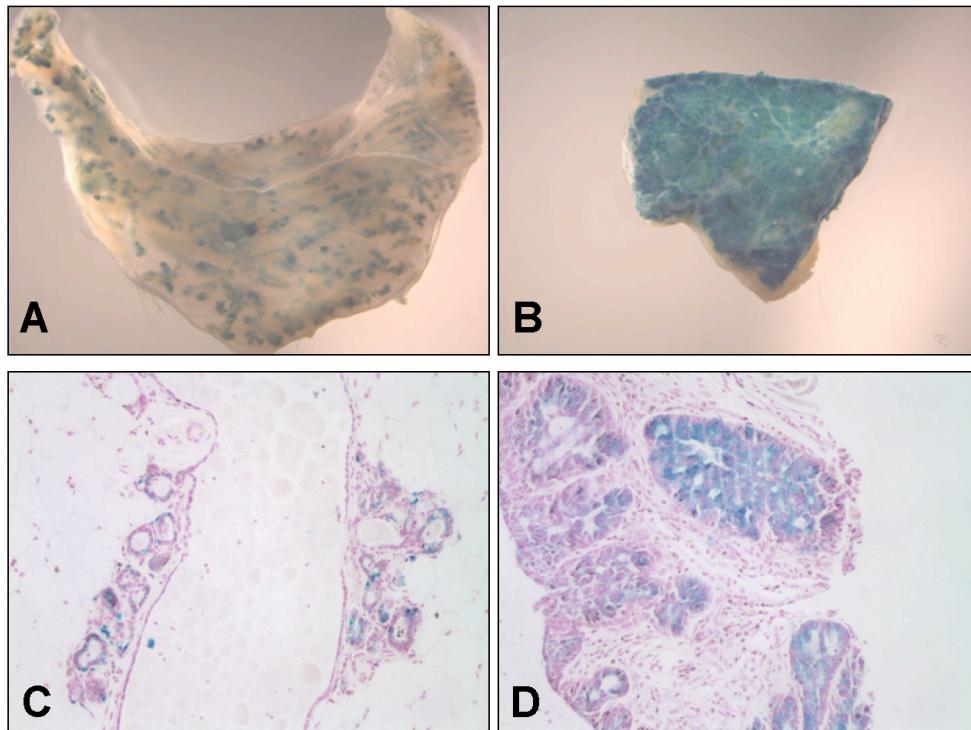


Figure 6: $lacZ^+$ cells in *WAP-Cre;TN;Rosa-lox-lacZ* triple transgenic virgin females. A: mammary gland whole mount stained for $lacZ$ activity. B: tumor whole mount stained for $lacZ$ activity. C,D: $lacZ^+$ hyperplastic alveolar cells (C) and $lacZ^+$ tumor cells (D). Within the tumor, only epithelial cells are $lacZ^+$, and all stromal cells are $lacZ^-$.

Figure 7.

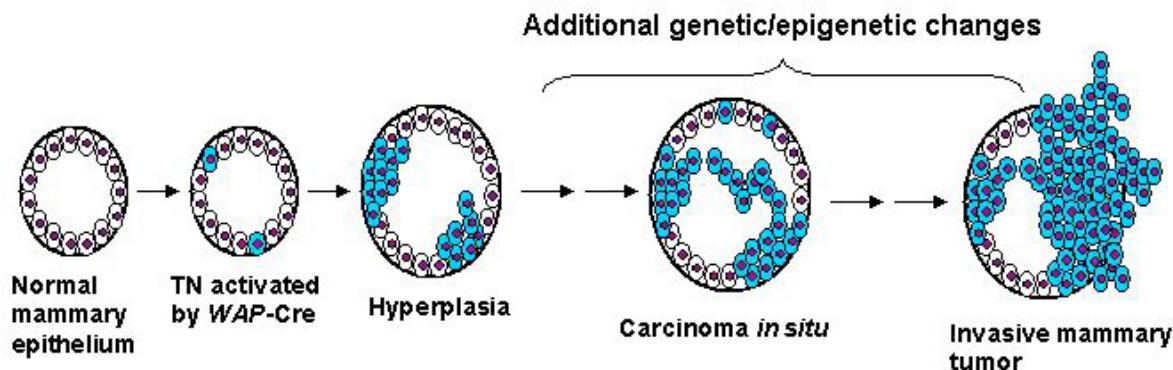


Figure 7. Current working model of tumorigenesis in *WAP-Cre;TN* virgin female mice.